AD_____

GRANT NUMBER: DAMD17-94-J-4336

TITLE: Genetic Instability and Checkpoint Genes: Insights from a Single Eucaryote (S. Cerevisiae) for Human Breast Cancer

PRINCIPAL INVESTIGATOR: Ted A. Weinert, Ph.D.

CONTRACTING ORGANIZATION: University of Arizona
Tucson, AZ 85719

REPORT DATE: October 1996

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1996	Final (16 Sep 94 - 15 Sep 96)

4. TITLE AND SUBTITLE
Genetic Instability and Checkpoint Genes: Insights from a Single Eucaryote (S. Cerevisiae) for Human Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4336

6. AUTHOR(S)
Ted A. Weinert, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Arizona
Tucson, AZ 85719

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

We study genes involved in cell cycle controls called checkpoints that block eucaryotic cell division after DNA damage. Checkpoint genes are relevant to breast cancer for several reasons, each related to aims of this proposal. Our first aim related to the fact that cancer cells and human checkpoint mutant cells exhibit high levels of genomic instability. We have characterized genomic instability in yeast cells and find an association between checkpoint mutations and chromosome loss and mitotic recombination. More importantly, we have identified and characterized an instance of genome instability (we call "unstable 7") useful in understanding how genomic instability can arise in checkpoint mutants.

Our second goal was to isolate human checkpoint genes, a task we were unsuccessful in probably because checkpoint gene homologs have only low sequence identify across species. We have undertaken a study of how one gene, *TEL1*, of low sequence homology to *MEC1* does suppress *mec1* checkpoint mutant defects. We have found suppression of damage-sensitivity, and of defects in transcriptional regulation and in cell cycle arrest. These studies provide a means of determining the order of gene function as well as of developing experimental conditions by human genes may complement yeast checkpoint mutation

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	25
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

TW In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

TW In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Ted A Wenert

PI - Signature Date

Table of Contents

5. Introduction

Checkpoint controls delay the cell cycle at specific cell cycle stages (e.g. G1, G2) after DNA damage (reviewed in Hartwell and Kastan, 1994). The delay provided by checkpoints provide time for DNA repair, and prevent genomic instability that may arise when damaged cells divide unchecked. Checkpoint genes are relevant to cancer in general and to breast cancer in particular for two reasons. First, checkpoint defects appear to contribute to cancer. Cancer progression is generally a multistep process in which cells accumulate mutations, each progressively allowing the cancer cell to grow under conditions where normal cells do not. One source of mutations occurs through genomic instability, a broad term meant to encompass any process which causes a mutant cell to rapidly accrue mutations or rearrangements. When checkpoint controls are defective, the mutant cells do not halt cell division when damaged, and progression with damage leads to further damage and rearrangements of chromosomes. This in turn can result in genetic alternations and progressively more severe cancer cell phenotypes (e.g. metastasis).

That checkpoint mutations coorelate with an acceleration of cancer cell phenotypes gains support from studies of two human genes p53 and recently ATM (Savitsky et al., 1995) that are both checkpoint genes. p53 is a gene long recognized as key in many cancer types. It is mutant in over 50% of all cancers ((Hollstein et al., 1991) and plays a prominent role in the G1 checkpoint arrest after damage (Kastan et al., 1991; Kuerbitz et al., 1992). ATM, for ataxi telengiectasia mutant, was identified by positional cloning (Savitsky et al 1995), and has a high association with several types of cancer including breast cancer (Swift et al., 1991; see ref. #). ATM proves to be related to our *MEC1* gene by both functional and protein sequence criteria (Figure 1, appendix). Both ATM and *MEC1* encode putative lipid or protein kinases (Hunter, 1995). Defects in either p53 or ATM, or in the yeast *MEC1* gene, lead to genomic instability (Livingston et al., 1992; Yin et al., 1992; Friedberg et al., 1995.), a phenotype probably related to the association of p53 and ATM mutations to cancer. In addition, the functional homology between human ATM gene and the yeast *MEC1* genes strengthens the view that understanding yeast controls will provide insights into understanding defective controls in cancer cells (Tugendrich et al., 1994).

Our goals in this proposal were as follows. First, we are analyzing the relationship between checkpoint gene functions and four types of genomic instability in *Saccharomyces cerevisiae*, a simple eucaryotic cell. We have now extended this study to analysis of an instance of genomic instability we call "unstable 7", which has many features suggesting it may be useful in understanding genomic instability in checkpoint mutant cells. Second, we wanted to isolate human checkpoint genes by complementation. Our

efforts have not been successful in this regard. To develop new strategies, we have turned to a detailed analysis of a *MEC1*-like gene, called *TEL1*, that can partially suppress *mec1* mutant defects. These studies will provide some information on order of gene function in checkpoint pathways. This studies of *TEL1* may also identify experimental conditions to identify human checkpoint genes by complementation. We anticipate these studies will further our basic knowledge of checkpoint gene function and enhance our understanding of mechanisms of genomic instability in tumor cells.

Our refined goals are:

Genomic Instability

1) Develop an assay of chromosome loss and mitotic recombination in the presence of checkpoint mutant genes. This assay is based on use of YAC artificial chromosomes, a stable but nonessential chromosome to the cell.

2). Develop an assay of point mutations in presence of checkpoint mutant genes.

3). Develop an assay of translocations between a YAC artificial chromosome and bona fide chromosome.

4) Develop an assay of gene amplification.

5) Analyze an instance of genomic instability- "unstable 7".

Human Checkpoint Genes

6) Study the role of *TEL1* and suppression of *mec1* mutant defects.

7)Isolate human checkpoint genes by complementation of yeast mutants.

6. Experimental Methods and Preliminary Results:

Our progress to date includes the following, addressing each research objective stated above.

Part1- Genomic instability

1). Chromosome loss and mitotic recombination assays: We modified the YAC artificial chromosome (Sears et al., 1992) as described in our proposal. This required extensive molecular cloning to insert appropriate selectable markers in the ends of the YAC (Figure 2, appendix). We then transferred these modified YAC into checkpoint mutant strains by karyogamy crosses (mating that allows transfer of only a chromosome into a recipient nucleus).

We found that all our checkpoint mutant strains showed elevated chromosome loss and recombination, especially *mec1* mutants. Unfortunately, the YACs were relatively unstable in the original strain, limiting the utility of this system. For example, we were unable to stably

introduce the two YAC chromosomes into the *mec1* strain; the only clones we could isolate were rearranged by the time we detected chromosome transfer (see Table 1, appendix). Our YAC-based chromosome loss system showed relative high instability (loss rate of 10^{-2} per cell division compared to loss rate of 10^{-5} per cell division for a bonafide chromosomes; (Hartwell and Smith, 1985) even in a checkpoint normal cell (though much less than in a *mec1* mutant)!

We had proposed two experimental approaches to resolve this issue. We are introducing our checkpoint mutations into a yeast strain in which the YAC's are stable (when those cells have intact checkpoint genes). I have contructed a *mec1 ts* allele linked to a selectable marker (*HIS3*), and am now introducing this allele into the S288C background (data not shown). Other checkpoint mutations will be introduced into S288C by using null mutations of checkpoint genes we have contstructed (Lydall and Weinert, 1995; submitted). If the S288C strain still proves difficult to work with, we still plan to use a second chromosome loss and recombination system based on a haploid strain containing an extra copy of chromosome 7 we used previously to characterize *rad9* (Weinert and Hartwell, 1990; and described further below).

Our preliminary results indicate that *mec1* mutants do show a tremendous instability of YACs, far greater than in our other strains (Table 1, appendix). This is consistent with *MEC1* having a key role in DNA replication compared to other checkpoint genes, a conclusion we and others have come to from other lines of research (Kato and Ogawa, 1994; Paulovich and Hartwell, 1995).

2). Point mutation assays:

We have analyze point mutations by two systems; conversion of $CAN1^S$ to $can1^r$ (selected for by resistance to the drug canavanine conferred by the $can1^r$ allele) and conversion of SUP4 to mutant SUP4 (selected by loss of ochre suppression; Pierce et al., 1987). Both assays indicate that our checkpoint mutants do not confer any significant "mutator" phenotype as assayed by point mutation rates, either spontaneous or in the presence of DNA damaging agents (data in Table 2, appendix for $can1^r$ system only).

3). Translocation assays:

We attempted to develop an assay of translocations by cloning a yeast gene into the human ALU sequence, to be placed back into the YAC chromosome and assayed for mobility. We completed initially cloning of *URA3* yeast gene into an ALUI sequence. The genomic instability assay in different strain backgrounds has caused us problems as discussed above, and the translocation assay will not be pursued until those other studies are completed.

4). Gene amplification assays:

We were going to generate a *ALU::HIS3* clone to being attempts to look at gene amplification. We had difficulty with the basic YAC system, and have therefore delayed development of a gene amplification assay. We have now identified gene amplification in the unstable 7 project described below. Once the region on chromosome 7 that is amplified has been defined, we will introduce a dosage-sensitive gene into that region. We plan to use the *HIS3* gene in combination with the drug scheme that selects for cells with a higher *HIS3* gene copy number.

5). The unstable chromosome 7 project

We have identified an instance of chromosome instability in *rad9* mutants we think will be very informative for genomic instability. A chromosome 7 disome (Figure 3, appendix; strain described in Weinert and Hartwell, 1990) allows us to select for loss of a marker at the end of the chromosome. This system was designed initially to identify chromosome loss and mitotic recombination events. It now proves to be an exceptionally useful tool to study other forms of genomic instability. I describe our current results next.

A. Genetic analysis:

We noticed a chromosomal event that shows hallmarks of a breakage-fusion-bridge cycle (McClintock, 1984). The genetic identification of this form of genomic instability occurs as follows: The initial strain used a haploid rad9 strain with 2 copies of chromosome 7 (Figure 3A, appendix; Genotype of initial cell). One homolog (denoted "B" for bottom) has a dominant selectable gene *CAN1s* gene which we can select for the loss of by plating cells on medium containing canavanine. The other homolog (denoted "T" for top) does not have the *CAN1s* gene. The initial cell with an intact $CAN1^s$ gene dies when plated on media containing canvanine. The few cells that lose $CAN1^s$ survive (frequency of survivors; 10^{-4} in *rad9-* cells and 10^{-5} in *RAD+* cells). Most $can1^r$ cells have undergone chromsome loss, an equivalent number undergo the event described below, and some have undergone mitotic recombination.

Among the cells that are resistant to canvanine were cells that gave rise to colonies with extensive sectoring ("nibbled" or "sectored" colonies; Figure 3B, appendix. Sectoring is probably due to cell death). For two reasons we believed that the sectored colonies had an event that was related to DNA damage checkpoint controls. First, the sectored colonies occurred spontaneously at about 10x higher rate in *rad9-* than *RAD9+* cells. Second, these sectoring colonies were also inducible by x-irradiation (not shown). We therefore set out to determine the source of the chromosomal event.

B. Sectoring colonies show extreme secondary instability

We first characterized the genotype of the sectoring colonies and found, remarkably, that >90% had the same genotype, denoted in Figure 3C, appendix. They appeared to have lost all DNA distal to the *TRP5* locus (shown as a dotted line). We thought initial that they were mitotic recombinants homozygous for the left arm of the chromosome. Subsequent analysis indicated otherwise.

The sectored colonies proved to be themselves extremely unstable genetically. This was identified by propagating the cells further on unselected medium. We surmised the following: consider an initial cell that has 2 chromosome 7s(T,B; Figure 3A, appendix). By selection, *CAN1*^{s} on the B homolog is lost. The sectored colonies contain T and a version of B we call here B*; they are T,B* (Figure 3B, appendix). When propagated these sectored cells with a "T,B*" genotype apparently undergo further events where either the T or B* chromosome is lost (frequency of secondary events in this experiment was:18% lose T; 11% lose B*; 71% retain T, B*; Figure 3D, appendix). Therefore, TB* cells undergo subsequent events at 1 in 3 cell divisions.

We have isolated 100's of TB* strains de novo from the initial disomic strain. The TB* cells always have the same characteristics with respect to genetic structure and instability (as shown in Figure 3C and D, appendix). We have not detected TB* strains arising from RAD+ strains; the few sectored colonies we detected had different genetic composition and did not give rise to subsequent instability as did the TB* in *rad9* mutants (data not shown).

C. Molecular analysis of unstable 7 (figures 4 and 5)

We wished to determine the molecular structure of B*. There are three key observations in deduction of the structure of B*. First, in strains undergoing the instability, we identified a chromosome that is physically larger than the initial chromosome 7 (Figure 4, appendix; note lane 4 in particular). We identified the larger chromosome using pulsefield gel electrophoresis that separates chromosomes by size on agarose gel matrix (Schwartz and Cantor, 1984). Most sectored colonies actually have the gel pattern shown in lane 4 (>90%). (The six cell preparations in Figure 4 derive from a non-random group of sectored colonies that had different genetic properties.) The starred bands represent extra bands corresponding to an unstable 7. That these extra bands are from chromosome 7 was demonstrated by hybridization of radio-labeled molecular probes from chromosome 7 (probe C, right panel). Furthermore, molecular analysis of the B* chromosomes verified that DNA genetically undetectable from the chromosome (see Figure 3C) was in fact <u>not present</u> on the unstable B* chromosomes. For example, the B* chromosome in

Figure 4, lane 4 hybridizes to a probe containing sequence C, so it contains the right arm of chromosome 7, but it does not hybridize to probe A, so it does not contain the left are of chromosome 7. The shorter B* chromosomes in Figure 4 also have the right arm but not the left arm of chromosome 7. (The band of lower molecular weight that hybridizes in the center and right panels is on chromosome 3, a control detected by using a probe, the *LEU2* gene, specific for chromosome 3.)

It thus appears that the B* chromosome has physically lost DNA. Yet, the truncated chromosome usually has the mobility of a larger chromosome by pulsefield gel electrophoresis.

We have identified molecularly the region of the breakpoints of the unstable chromosome, narrowed to a region of 10-50kb (not shown). This was accomplished using yeast DNA cloned into a bank of lambda phages (Riles et al, 1993.). Most unstable 7 chromosomes have similar though not identical breakpoints (judging from the intensity of the hybridization signals). The breakpoints appear heterogeneous but cluster in this one region of the chromosome. This site appears, therefore, to be some sort of stabilization site and/or selectively broken site. We do not now know which interpretation is correct.

D. Defining the structure of B*: Use of "Stretch DNA" technology

We needed a cytogenetic test of yeast chromosome structure. Yeast chromosomes in metaphase are unfortunately not visible like yeast chromosome structure is not visible, though a different technique to probe structure was required. During a recent Gordon Conference I had the good fortune to learn of a new technique to visualize chromosomes and specific sequences by a method termed "stretch DNA". Such a test is now possible, in collaboration with Ulli Weier at UC Berkeley. He has a method to lay out (stretch) intact chromosomes and identify the relative distances between sequences using flourescence probes (FISH; Weier et al., 1995). His method allowed us to test the physical structure of the B* chromosome.

We sent him gel-isolated (see Figure 4) unstable and stable chromosome 7. He prepared "stretched chromosomes"and studied their structures using fluorescently labeling DNA probes (FISH). To prepare "stretched chromosomes" he applies the isolated chromosomes to a glass slide, and upon dehydration the DNA molecule stretches across the slide, remaining intact. These "stretched chromosomes" are subjected to FISH hybridization using probes to specific regions of chromosome 7 (obtained from the yeast lambda clones; Riles et al., 1993).

We have received data on structure of the stretched chromosomes. The B* chromosomes structures show three unexpected features (Figure 5, appendix). First, they do contain two right chromosome ends (identified by FISH probe to a right-end sequence; Figure 5, righthand image). B*

therefore appears to be a head to head fusion of chromosome 7. Second, they identified only a single centromeric sequence (Figure 5, lefthand image). The B* structure is, surprisingly, not a dicentric. Third, another surprise; some 100-200 kb DNA circles were found as well. These circles of DNA do contain chromosome 7 sequences, in fact from the right arm (region C). The fiber analysis, with 50-100 fibers analyzed in each sample was quantitative; all B* chromsomes had 2 signals with a right end probe, each near the fiber ends as expected from the physical distance of the sequence to the chromosome end. Each normal chromosome had one signal near an end, and both samples showed only a single signal to a centromeric probe.

E. Model

A model to explain these observations is shown in Figure 6 (appendix). We hypothesized that an initial rare event lead to a chromosome break. The checkpoint-defective cells continue through the cell cycle, rereplicate the broken chromosome, generating two truncated sisters (with sequence B near break points). These can then fuse, recombine with other homolog (not shown) be propagated as is or be lost. If the two fuse, this structure can undergo breakage-fusion-bridge cycles and rearrangements, generating a structure shown in Figure 6 (McClintock, 1984).

Many features of our data are consistent with this model; rare initial event inducible by radiation; high frequency secondary chromosome loss events; high frequency in a checkpoint mutant cell; structures shown in Figure 5.

F. Future direction:

1. The instability of B* , which is a moncentric chromosome, is yet to be explained. Instability may be related to the circles of DNA found co-migrating with B*. These circles remain enigmatic.

The entire sequence of chromosome 7 is now in the GCG database. We will perform DNA sequence analysis to determine the following:

-Are there sequence features near the breakpoint that account for why these structures are preferentially recovered? Are there inverted repeat sequences or telomere-like sequences in this region?

-Are there sequences near chromosome ends that can explain the generation of circles- for example are their direct repeats in region C?

2. We have also identified a 1400kb B* chromosme that we will analyze by stretch DNA methodology. This chromosome has the correct size to be a complete dicentric, which can be tested directly by FISH probes to centromere DNA.

3. We have identified sequences proximal to the breakpoint that are amplified (determined by quantitative southern analysis; data not shown). We will insert a dosage-sensitive gene, *HIS3*, into this region and determine if we can use this as a system to genetically detect gene amplification.

4. We have contructed *rad9ts* chromosome 7 disomic strains to ask if reactivation of the checkpoint will limit propagation of the B*-containing cells. This experiment tests if the checkpoint controls can limit these types of events once the abnormal B* structure is generated.

Part 2- Isolation of Human checkpoint genes.

The idea that checkpoint genes are conserved in humans and yeast has been verified - the ATM gene from humans is very similar functionally and by protein sequence (though at the low end- 20% identical, 50% similar; Savitsky, et al. 1995) to that of our *MEC1* gene (see Figure 1). Others who have the ATM gene are attempting complementation of *mec1* mutants or test of dominant negative activity of *MEC1* in human cells (Steve Friend, Seattle, Wa; Mike Kastan, Baltimore, Md; personal communications). Therefore, the existence of human counterparts to at least some yeast checkpoint genes seems highly likely.

Complementation of yeast checkpoint mutants by human cDNAs has not, in our hands, been a successful strategy. Others have tried and failed as well (see Plon et al, 1993). The reason for these failures may be in that homologs bear the low level sequence homology. this is true for both human ATM and yeast *MEC1* genes, as well as for four pairs of homologous genes between budding and fission yeasts (@20% identity; 45% similarity; see Lydall and Weinert, 1995, 1996). Though functions may be conserved, molecular contacts with other proteins may have diverged signficantly to make cross function unlikely. The only strategy we will now pursue is through the use of homology identities between our yeast genes and cDNA sequences from random human cDNA sequences, called EST sequences. Any putative match will be followed up by collaboration with those who identified the putative matching EST sequence.

Part3- Analysis of *TEL1*, a yeast *MEC1*-like and ATM like gene.

In the interim, we have initiated a project to characterize one gene that bears low level homology with *MEC1*, a yeast gene called *TEL1* (Greenwell et al., 1995; Morrow et al., 1995; Sanchez et al., 1996). The analysis described below has two goals. The primary goal is to determine the order of gene function, a goal in which we have made significant progress. A secondary goal is to identify a complementation strategy, a checkpoint-related phenotype, that may detect genes with lower amount of function.

We have undertaken a study of the role of *TEL1* in the cells responses to DNA damage. The principle questions and preliminary findings are the following: To what extent can *TEL1* substitute for *MEC1*? The answer is, a little. Second, does *TEL1* provide a useful genetic tool to order gene functions inthe checkpoint pathway? The answer appears to be "yes". Third, by learning how *TEL1* can substitute for *MEC1* can we develop a sensitive strategy to identify other human genes that have low level of homology to our checkpointgenes? We do not yet know the answer to this question.

The model of gene interactions we are currently testing is shown in Figure 7A. There is considerable evidence in favor of this model, including genetic, molecular and biochemical data. For example, *MEC1* and *TEL1* share sequence similarity (Greenwell et al., 1995; Morrow et al., 1995; Sanchez et al., 1996); *TEL1* can restore some responses to DNA damage in *mec1* mutants (Morrow et al., 1995; Sanchez et al., 1996; see below); *RAD53* may act downstream of *MEC1* and *TEL1* because Rad53p is phosphorylated in vivo after damage in a *MEC1* or *TEL1*-dependent manner (Sanchex et al., 1996; Sun et al., 1996); *DUN1* acts specificallyin transcriptional regulation (Zhou and Elledge, 1993); PDS1 acts specifically in G2/M arrest (Yamamoto et al., 1996)

Our current strategy is to evaluate how *TEL1* restores activity in *mec1* mutants. Restoration of activity requires that we overexpress *TEL1* from a high copy plasmd and monitor each of four responses; restoration of DNA damage resistance, restoration of G2/M arrest, restoration of transcriptional regulation of damage-inducible genes, and restoration of viability to *mec1* null mutants. The restoration of damage sensitivity is significant, and may be useful in some form in screens of human checkpoint genes (Morrow et al., 1995; Sanchez et al., 1996; data Figure 7B). We have recently shown that *TEL1* can restore transcriptional induction after damage by the alkylating agent MMS (Figure 7C), and that *TEL1* can restore partially cell cycle arrest (Figure 7D).

A key question is whether any of these *TEL1*-mediated responses occur by activation of checkpoint pathways , or do they occur by some unknown and therefore uninterpretable pathway activated by overexpression. Gratifyingly, we found that transcriptional induction by *TEL1* requires *DUN1*, suggesting this response proceeds by the defined pathway (Figure 7C). Second, suppression of lethality is DUN1 dependent (data not shown). Third, overexpression of *TEL1* causes cell cycle arrest that requires *PDS1* (data not shown). Interestingly, overexpression of *TEL1* does not require damage nor *RAD9*, suggesting that overexpression causes a constitutive activation of the checkpoint pathway leading to arrest. We are in the process of analyzing this network of genes in more detail to test the hypothetical pathway. Overexpression of *RAD53*, *PDS1*,

and *DUN1* all cause phenotypes potentially useful in genetic epistasis experiments done in combination with loss of function alleles in any of the five genes shown.

7. Conclusions

First, analysis of genomic instability in checkpoint mutant cells remains an incomplete task. Our initial tests of *mec1* suggest it leads to high rates of mitotic recombination, and we will pursue this observation using the YAC recombination system and *mec1ts* allele we have constructed. The point mutation frequencies do not seem to be elevated in our mutants. We will try to generate a system to identify gene amplification using the sequences on chromosome 7 that can be amplified.

Our most significant progress remains with the genomic instability of chromosome 7, a phenomenon that we now call unstable 7. This event may provide us the opportunity to study genomic instability occuring by the breakage-fusion-bridge cycle as proposed by McClintock (1984). Further analysis of the unstable 7 chromosome should provide additional insights on the role of checkpoint genes in genomic stability.

We are still uncertain as to whether human checkpoint genes can be identified by complementation of yeast mutants. Detailed analysis of *TEL1* may provide tests of order of gene function as well as clues to sensitive assays that may be useful to identify human checkpoint genes.

8. References:

Friedberg, E. C., Walker, G. C. and Siede, W. (1995). DNA Repair and Mutagenesis.

Greenwell, P. W., Kronmal, S. L., Porter, S. E., Gassenhuber, J., Obermaier, B. and Ptes, T. D. (1995). TEL1, a Gene Involved in controlling telomere length in S.cerevisiae, is homologous to the human ataxia telangiectasia gene. Cell *82*, 823-829.

Griffiths, D. J. G., Barbet, N. C., McCready, s., Lehmann, A. R. and Carr, A. M. (1995). Fission Yeast rad17: a homolog of budding yeast RAD24 that shares regions of sequence similarity with DNA polymerase accessory proteins. EMBO J *14*, 101-112.

Hartwell, L. H. and Kastan, M. B. (1994). Cell Cycle Control and Cancer. Science *266*, 1821-1828.

Hartwell, L. H. and Smith, D. (1985). Altered fidelity of mitotic chromome transmission in cell cycle mutants of *S. cerevisiae*. Genetics *110*, 46.

Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C. C. (1991). p53 Mutations and Human Cancers. Science *253*, 49-53.

Hunter, T. (1995). When is a lipid kinase not a lipid kinase? When it is a protein kinase. Cell *83*, 1-4.

Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B. and Craig, R. W. (1991). Participation of p53 protein in the Cellular Response to DNA damage. Canc. Res. *51*, 6304-6311.

Kato, R. and Ogawa, H. (1994). An essential gene, ESR1, is required for mitotic cell growth, DNA repair and meiotic recombination in Saccharomyces cerevisiae. Nucl.Acids.Res. *22*, 3104-3112.

Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V. and Kastan, M. B. (1992). Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Nat'l. Acad. Sci. (USA) *89*, 7491-7495.

Livingston, L. R., White, A., Sprouse, J., Livanos, E., Jacks, T. and Tisty, T. D. (1992). Altered Cell Cycle Arrest and Gene Amplification Potential Accompany Loss of Wild-Type p53. Cell *70*, 923-935.

Lydall, D. and Weinert, T. (1995). Yeast Checkpoint Genes in DNA Damage Processing: Implications for Repair and Arrest. Science *270*, 1488-91.

Lydall, D. and Weinert, T. (1996). From DNA damage to cell cycle arrest and suicide: a budding yeast perspective. Curr. Op. Gen. Dev. *6*, 4-11.

McClintock, B. (1984). The significance of responses of the genome to challenge. Science *226*, 792-801.

Morrow, D. M., Tagle, D. A., Shiloh, Y., Collins, F. S. and Hieter, P. (1995). TEL1, an S.cerevisiae Homolog of the Human Gene Mutated in Ataxia Telangiectasia, is Functionally Related to the Yeast Checkpoint Gene MEC1. Cell *82*, 831-840.

Paulovich, A. G. and Hartwell, L. H. (1995). A Checkpoint Regulate the Rate of Progression through S phase in S.cerevisiae in Response to DNA Damage. Cell *82*, 841-847.

Pierce, M. K., Giroux, C. N. and Kunz, B. A. (1987). Development of a yeast system to assay mutational specificity. Mutation.Res. *182*, 65-74.

Plon, S. E., Leppid, K., A., Do, H.-N. and Groudine, M. (1993). Clong of the human homolog of the CDC34 cell cycle gene by complementation in yeast. Proc.Natl.Acad.Sci.U.S.A. *90*, 10484-10488.

Riles, L., Dutchik, J. E., Baktha, A., mcCauley, B. K., Thayer, E. C., Leckier, M. P., Braden, V. V., Depke, J. E. and Olson, M. V. (1993). Physical maps of the six smallest chromosomes of Saccharomyces cerevisiae at a resolution of 2.6 kilobase pairs. Genetics *134*, 81-150.

Sanchez, Y., Desany, B. A., Jones, W. J., Liu, Q., Wang, B. and Elledge, S. (1996). Regulation of *RAD53* by the *ATM*-Like Kinases *MEC1* and *TEL1* in Yeast Cell Cycle Checkpoint Pathways. Science *271*, 357-360.

Savitsky, K., Bar-Shira, A., Gilad, S. and al., e. (1995). A single Ataxia Telangiectasia Gene with a Product Similar to PI-3 Kinase. Science *268*, 1749-53.

Schwartz, D. C. and Cantor, C. R. (1984). Separation of Yeast Chromosome-sized DNAs by Pulsed Field Gradient Gel Electrophoresis. Cell *37*, 67-75.

Sears, D. D., Hegemann, J. H. and Hieter, P. (1992). Meiotic Recombination and segregation of human-derived artificial chromosomes in Saccharomyces cerevisiae. Proc.Natl.Acad.Sci. U.S.A. *89*, 5296-5300.

Sun, Z., Fay, D. S., Marini, F., Foiani, M. and Stern, D. F. (1996). Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways. Genes Dev. *10*, 395-406.

Swift, M., Morrell, D., Massey, R. B. and Chase, C. L. (1991). Incidence of cancer in 161 families affected by ataxia telangiectasia. N.Engl.J.Med. *325*, 1831-1836.

Tugendrich, S., Bassett, D. E., McKusick, V., A., Boguski, M. S. and Hieter, P. (1994). Genes Conserved in yeast and humans. Human Mol. Genetics *3*, 1509-1517.

Weier, H.-U., Wang, M., Mullikin, J. C. and al., e. (1995). Quanitative DNA fiber mapping. Human Mol. Genetics *4*, 1903-10.

Weinert, T. A. and Hartwell, L. H. (1990). Characterization of *RAD9* of *Saccharomyces cerevisiae* and Evidence that Its Function Acts Posttranslationally in Cell Cycle Arrest after DNA Damage. Mol. Cell. Biol. *10*, 6554-6564.

Yamamoto, A., Guacci, V. and Koshland, D. (1996). Pds1p, an inhibitor of Anaphase in Budding Yeast, Plays a critical role in the APC and Checkpoint Pathways. J.Cell. Biol. *133*, 99-110.

Yin, Y., Tainsky, M. A., Bischoff, F. Z., Strong, L. C. and Wahl, G. M. (1992). Wild-Type p53 Restores Cell Cycle Control and Inhibits Gene Amplification in Cells with mutant p53 Alleles. Cell *70*, 937-948.

Zhou, Z. and Elledge, S. J. (1993). DUN1 encodes a protein kinase that controls the DNA damage response in yeast. Cell *75*, 1119-1127.

Figure 1 Similarity of ATM, MEC1 and other PI3 kinases

An alignment of genes in the PI3 kinase family. From Meyn, S 1995. Cancer Research 55, 5991-6001. Ataxia-telangiectasia and Cellular Responses to DNA Damage. Shown are the ATM gene; DNA-PK gene that encodes a mammalian protein kinase activated by DNA damage; MEI41, the functional Drosophila homolog of the yeast MEC1; the yeast Tel1p; RAD3, the fission yeast homolog of MEC1; and MEC1. The identity in the solid box is 60-70% similarity, in the shaded box is 40-50% similarity, and the rest of the sequences have less then 20% similarity.

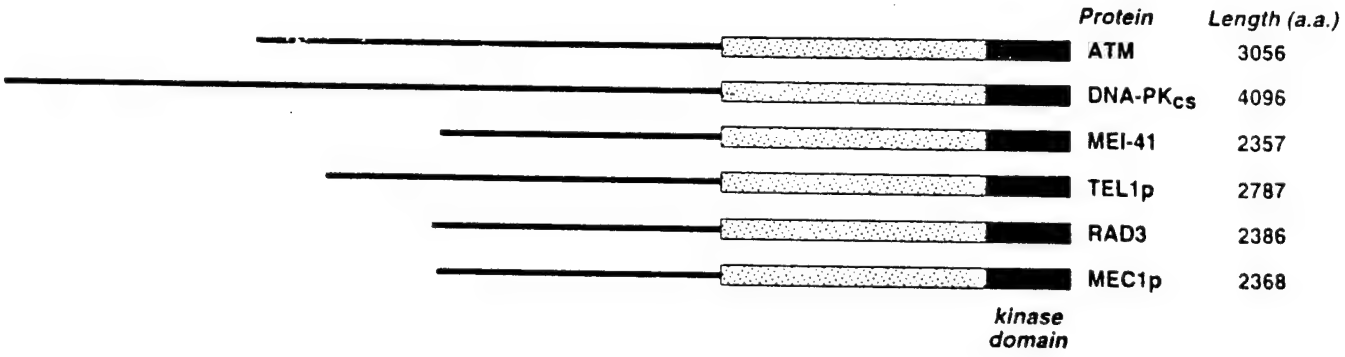


Figure 2, Yac chromosomes constructed to detect chromosome loss and recombination. These YACs were transferred into a ura3, trp1 lys2 cyhr strain. The lose of the dominant sensitivity allele CYH-S,that confirms sensitivity to cycloheximide allows selection of chromosomal events. Loss generates cells that are cyhr ura- Lys+ Trp+, and recombinants are cyh2r, Ura+ Lys+ Trp+.

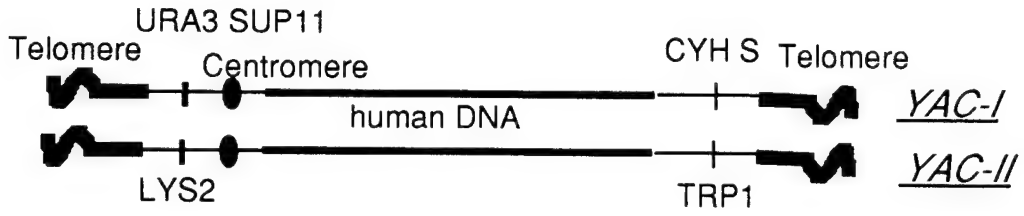


Table I Chromosome Loss and Recombination of YAC chromosomes in checkpoint mutants.

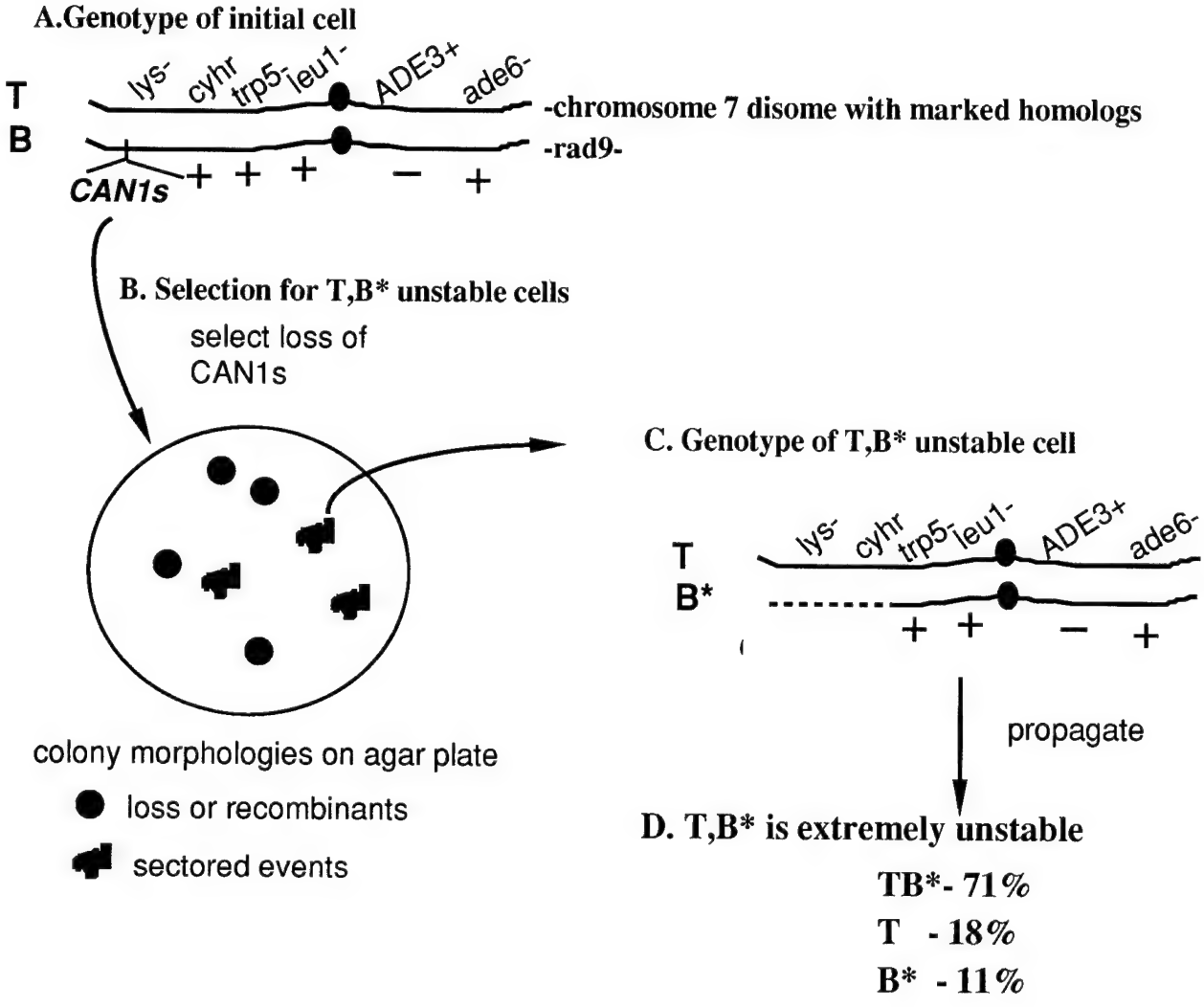
Strain	Loss Frequency ($x10^{-2}$) cyhr Ura- Lys+ Trp+	Recombination Frequency($x10^{-2}$) cyhr Ura+ Lys+ Trp+
MEC+(trial1)	0.7 +/- 0.9	0.3 +/- 0.1
(trail 2)	0.13 +/- 0.17	1.1 +/- 2.1
mec1	not determinable	49 +/- 28
mec2 (rad53)	23.4 +/- 17.2	10.2 +/- 13.2
rad9 (trail 2)	0.71 +/- 0.76	2.8 +/- 3.2

Table 2 Point Mutation Frequency in Checkpoint mutant strains.

Strain	Frequency $can1^R$ from $CAN1^S$
MEC+	3.9×10^{-5}
mec1	1.5×10^{-5}
mec2 (rad53)	3.8×10^{-5}
mec3	1.8×10^{-5}
rad9	4.4×10^{-6}
rad17	3.8×10^{-6}
rad24	4.2×10^{-5}

(average frequency from 5 samples)

Figure 3. Unstable 7



* Note: this event is not detected in RAD+ strains beyond the generation of sectored colonies. Upon propagation the cells acquire stable genotypes, and we have not detected extra chromosomes by pulsefield gel analysis in RAD+ cells.

Figure 4- Molecular analysis of B*

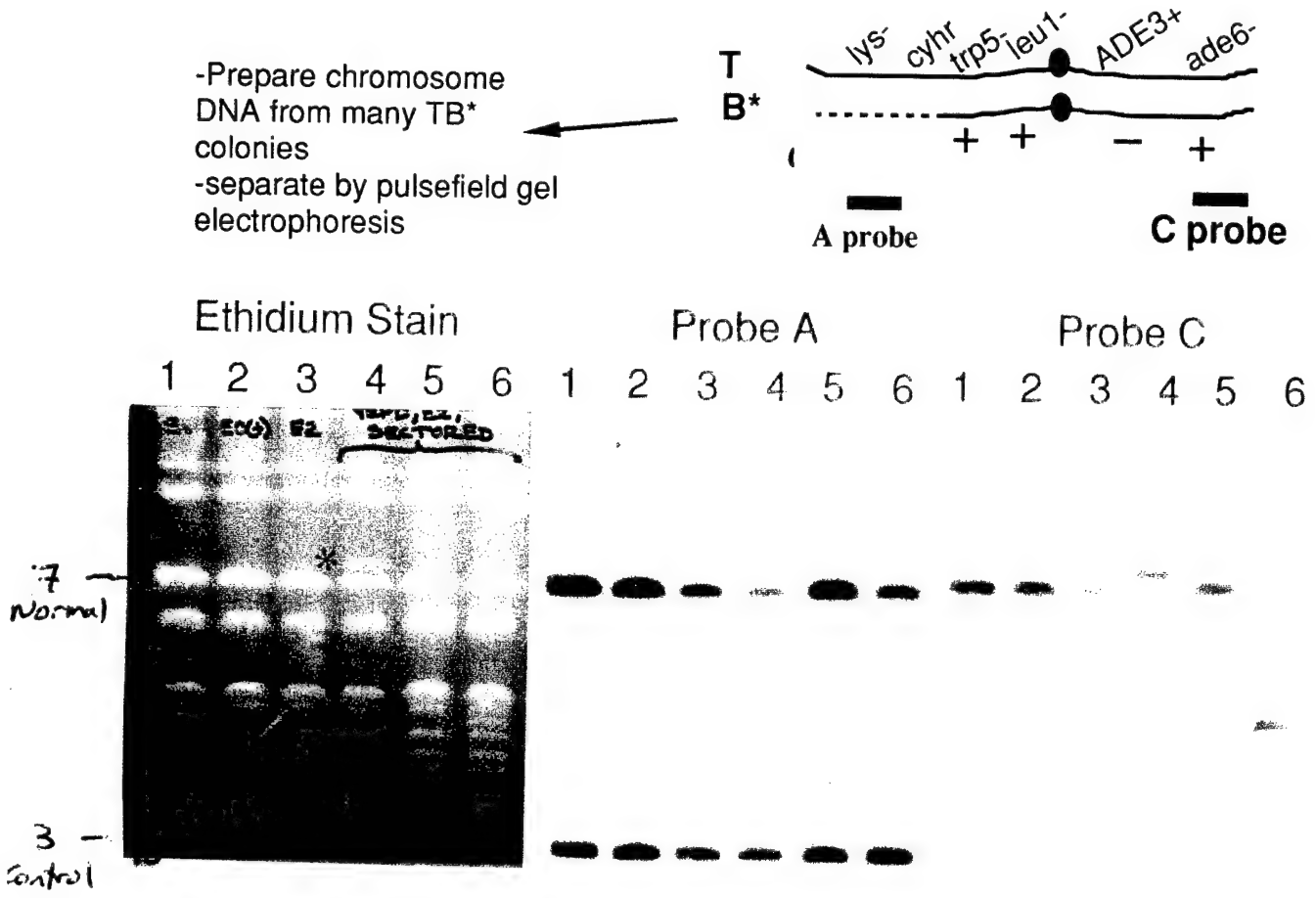


Figure 5. Structures of streched B* chromosomes.
left- hybridized with centromere probe- note white arrow.
right panel- hybridized with right-arm probe- note two black arrows.

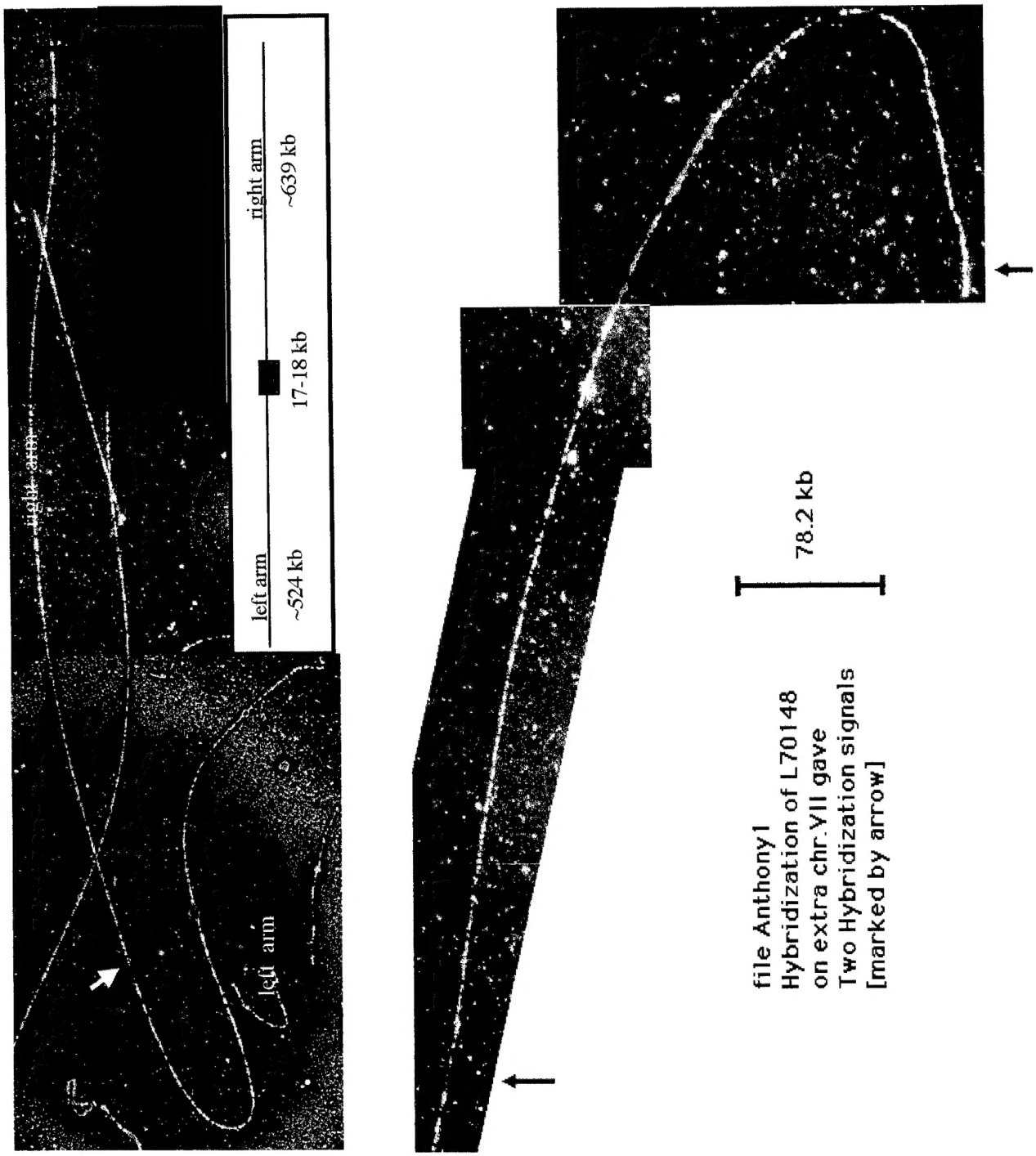
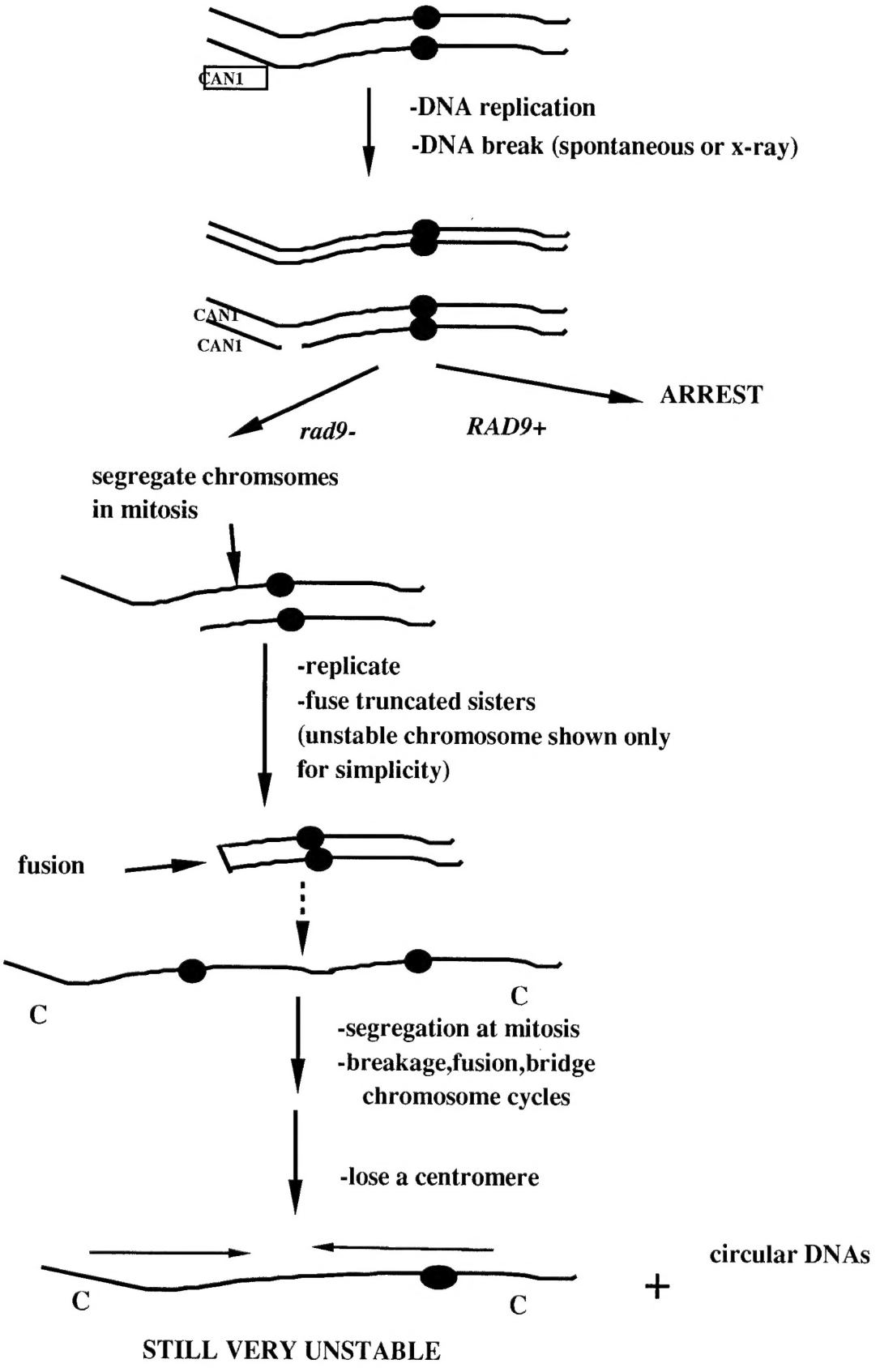


Figure 6. Model of Unstable 7



A. Hypothesis on Order of Gene Function

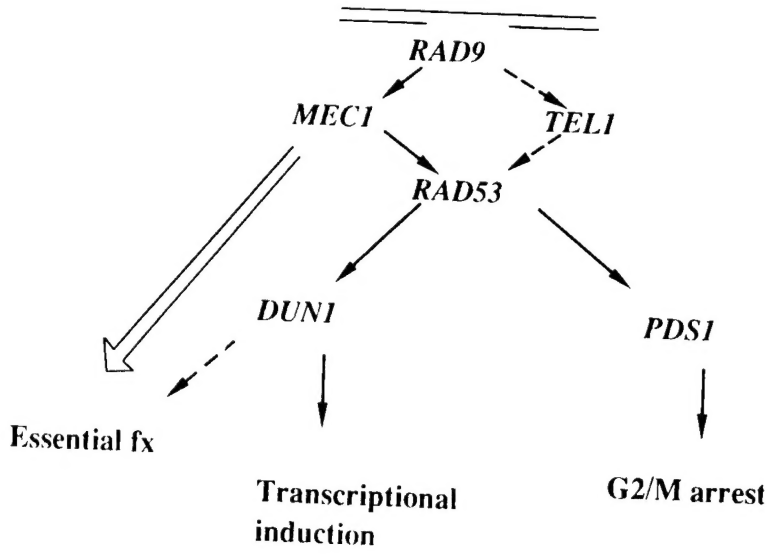
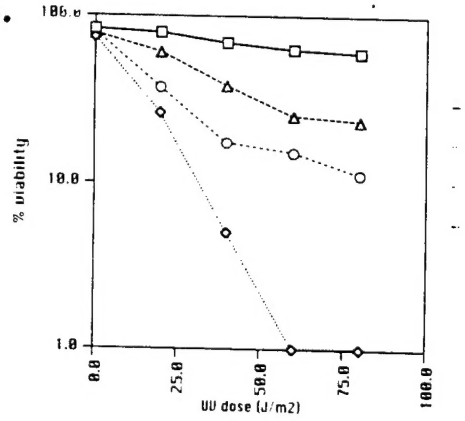


Figure 7- Analysis of Order of Gene Function

A. Model. DNA damage (top) activates RAD9 and then subsequent genes. Solid lines represent the normal pathway, dotted lines the pathway achieved upon overexpression.

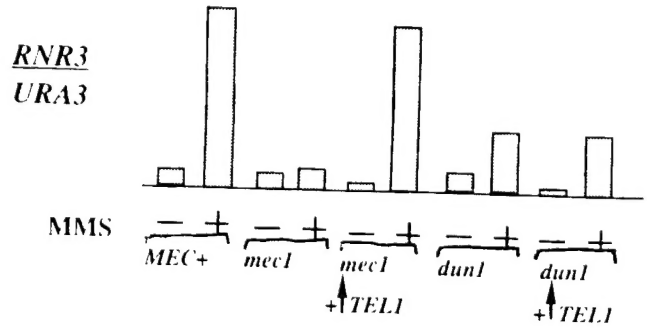
B.



B. Suppression of UV sensitivity of *mec1* mutants by overexpression of *TEL1*.

- □wildtype
- ◇*mec1* + vector
- ○*mec1* +*TEL1* (1 copy)
- △*mec1* + *TEL1* (high copy)

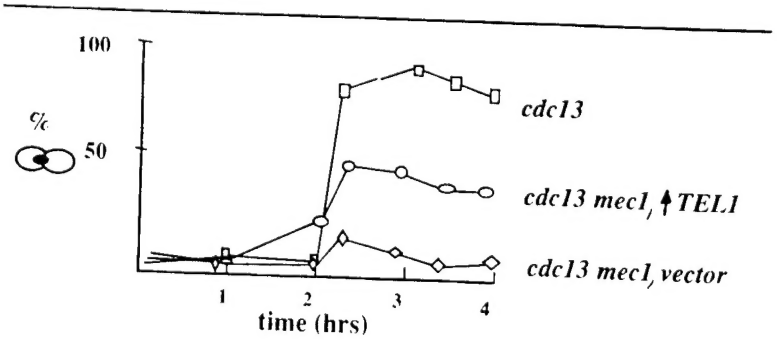
C.



C. *TEL1* restores transcriptional induction of a damage-inducible gene (*RNR3*) in *mec1*

-Relative levels of RNR3, determined by northern analysis, with and without DNA damage from 0.01% MMS, 4 hours. Induction is DUN1 dependent.

D.



D. *TEL1* restores partial cell cycle arrest to *mec1* mutants.

-Cells were synchronized in G1 with mating pheromone, released into the cycle at the restrictive temperaure (36) for *cdc13*,after washing out mating pheromone. *cdc13* mutation generates damage during S phase. Cells were fixed and stained for nuclear morphology. *cdc13* cells arrest to determine arrest. *cdc13* cells arrest, *cdc13 mec1* mutants do not, and *TEL1* partially restores arrest

Weinert, Ted A.
DAMD17-94-J-4336

(11) Personnel

Ted A. Weinert, P.I.
Anthony Admire, Research Specialist
Jane Ferguson, Research Specialist
Michael Nejad, Graduate Assistant
Elizabeth Little, Graduate Assistant
David Morse, Graduate Assistant
Krista Colmar, Undergraduate Assistant
Christine Emminger, Undergraduate Assistant
Alex Herskowitz, Undergraduate Assistant
Vesna Kaluza, Undergraduate Assistant
Karen Tanaka, Undergraduate Assistant